The Plymouth Student Scientist - Volume 02 - 2009 The Plymouth Student Scientist - Volume 2, No. 1 - 2009

2009

The Effects of Bisphenol A on in-vitro Cell Viability of Mammalian Cell Line by Neutral Red Assay

McGlinchey, A.J.

McGlinchey, A.J. (2009) 'The Effects of Bisphenol A on in-vitro Cell Viability of Mammalian Cell Line by Neutral Red Assay', The Plymouth Student Scientist, p. 25-31.
http://hdl.handle.net/10026.1/13850

The Plymouth Student Scientist
University of Plymouth

*The Plymouth Student Scientist, 2009, **2**, (1), 25-31*

The Effects of Bisphenol A on in-vitro Cell Viability of Mammalian Cell Line by Neutral Red Assay

Aidan J. McGlinchey

2009

Project Advisor: Awadhesh Jha School of Biological Sciences, University of Plymouth, Drake Circus, Plymouth, PL4 8AA

Abstract

Bisphenol-A (BPA), a known oestrogenic endocrine disruptor, has many effects in-vivo. However, most studies on its effects concentrate on this oestrogenic property and not on direct cytotoxicity. An in-vitro approach was taken here, showing that BPA has a dose-dependent effect on the viability of Chinese hamster ovary (CHO-K1) cells. Concentrations at which BPA directly generates cytotoxic effects were investigated by exposing CHO-K1 to BPA for 48 hours at 37^{o}C and their viability assessed by their ability to take up and retain the neutral red staining agent. Cells were exposed to tenfold dilutions of BPA from 1x10^{-4}M to 1x10^{-9}M and viability measured as a function of absorbance at 540nm wavelength. The greater the absorbance, the more neutral red was successfully taken up by the cells, indicating superior health and general viability. Resulting absorbances ranged from a mean of 2.435A (SEM 0.162) for 1x10^{-9}M BPA down to 1.924A (SEM 0.103) for 1x10^{-4}M BPA. A clear relationship between BPA concentration and reduction in absorbance emerged after exposure as assessed by one-way ANOVA. A one-way ANOVA performed on stacked results of 1x10^{-4}M to 1x10^{-6}M BPA and stacked results of 1x10^{-7}M to 1x10^{-9}M BPA revealed a highly statistically significant difference between these groups (p=0.000). This suggests that there is a cytotoxicity "threshold" between 1x10^{-7}M to 1x10^{-6}M BPA, over which a proportionately large increase in cytotoxicity is seen, compared to lower concentrations. Analysis of the difference between only the 1x10^{-7}M to 1x10^{-6}M by one-way ANOVA fell just short of the 95% confidence interval (p=0.058), showing that around these concentrations is where further work need focus to ascertain a highly accurate point of increased cytotoxicity. Whilst this "toxicity threshold" has been elucidated by these analyses, it must be noted that the other concentrations investigated most likely also displayed decreased viability compared to control medium. However, for reasons presented in this paper, the control measurements made were not reliable and so further control testing and comparison will be needed to provide much-needed perspective on the obtained results. The cytotoxic "threshold" of BPA revealed herein further calls for assessment of public health and investigation of exposure to this cytotoxic agent. Recommendations for further studies and the wider implications of the concentrations investigated here are also given in this paper.

Keywords: bisphenol A, neutral red, endocrine disruptor, in-vitro, cytotoxicity, public health.

ISSN 1754-2383 [Online]

1. Introduction

Bisphenol-A (BPA) is a planar molecule that mimics the structure of oestrogen and can activate oestrogen receptors in a manner 1:2000 the potency of normal 17β-estradiol (Bergeron et al., 1999). BPA has also been shown to act on thyroid hormone receptors (Moriyama et al., 2002) (Zoeller et al., 2005) and the monoaminergic nervous system (Negishi et al., 2004), amongst other effects.

BPA is used as a monomer in the manufacture of polycarbonate plastic worldwide and BPA itself is produced in an amount in excess of 6.4 billion pounds per year (vom Saal et al., 2005) and has been shown to leach out of polycarbonate plastic products (Krishnan et al., 1993). Worse, it has been shown that this leaching increases as the product ages. This has been demonstrated using laboratory cages for animals that leached BPA into distilled water (0.3µg BPA/L water) in new cages compared to (310µg/L) in aged cages (a 1033 fold increase), indicating that the longer we are in contact with polycarbonate products and the older those polycarbonate products are, the greater our exposure to BPA (Howdeshell et al., 2003). This is of particular concern to babies fed from polycarbonate bottles or foetuses whose mothers are exposed to BPA as the child will be exposed to BPA in high amounts (the placenta presents no barrier to BPA – (Ikezuki et al., 2002.)) BPA is also increasingly found in our natural environment, leaching from landfill sites and contributing up to 84% of the landfill site's oestrogenicity (Kawagoshi et al., 2003.) This leaching BPA in our environment, aside from other effects mentioned below, can even reduce the fertility of our soil by inhibiting the ability of nitrogen-fixing bacteria to function properly (Fox et al., 2007.)

Many studies have confirmed the oestrogenic and pathogenic effects of BPA in-vivo, mostly in rats and mice, but also in humans. These effects include, but are not limited to, aneugenic effects (Hunt et al., 2003), early onset of puberty (Honma et al., 2002) (Howdeshell et al., 1999), altered reproductive development (Nagel et al., 1997) (Kawai et al., 2003), obesity (Masuno et al., 2002), diabetes (Alonso-Magdalena et al., 2006), altered brain development (Ishido et al., 2004), immune interference (Sawai et al., 2003) (Yoshino et al., 2004), increased cancer cell growth (Vandenberg et al., 2007) (Murray et al., 2007) etc. However, the metabolism of bisphenol-A in a whole-organism setting can distort our understanding of its effects were such metabolism absent. In-vitro studies allow us to see the direct effect of the compound on cells as they are exposed to it in the absence of more complex metabolism for a clear view of its potential effects. Essentially, by using cells in culture, we eliminate the variation the in-vivo studies carry by removing the factor of the hosts' differing abilities to clear an insult of BPA (for example, studies in rats and humans are not comparable due to the presence, in rats, of enterohepatic circulation, greatly altering their processing of BPA).
Assessing the toxicological effects of BPA, as opposed to its oestrogenic effects, has not been the main focus of the literature published on BPA. Cytotoxic effects in addition to oestrogenic effects may well be extremely important in our understanding of the broader range of effects that BPA has on organisms. A mammalian cell line was chosen here to present our results in a manner that is as relevant to humans as possible. This also avoided lengthy ethical approval and would allow the end result to be repeatable without such hassle in a cell line that is well established with very little variation for maximal consistency in results and methods.

The neutral red assay was chosen because it assesses the lysosomal integrity of cells. As the integrity of the lysosome in cells is dependant upon a very wide range of healthy internal machinery, as opposed to a single or a few individual pathways, it provides a good overview of general cellular health as deficient systems inside the cell are likely to impact on the cells ability to retain the neutral red stain. The neutral red assay also results in easily measurable and useable data in the form of absorbances, enabling clear and concise comparison of the effects of agents on the health of the cell. These data can be directly analysed statistically, due to their numeric nature, providing a means of testing for significance as well as more basic comparison. Regarding the choice of cells, a mammalian cell line was chosen to maximise relevance to humans without having to source human cells and the ethical considerations that would involve. CHO-K1 cells have been used for many nutrient and genetic studies in the past and are also useful for viability assays such as this.

BPA has been shown to be metabolised quickly with a half life of around <6 hours (Volkel et al., 2002), after which it is known to be completely metabolised to bisphenol-A glucuronide (Ouchi et al., 2002.) However, BPA has been found at a median level of 3.1ng/ml in plasma of 37 pregnant women (Schönfelder et al., 2002), suggesting a relatively constant exposure at low levels, despite fast metabolism. If indeed, such low concentrations of BPA are being experienced by people as a whole, then it would be prudent to see whether, on a cellular level, such concentrations of BPA caused direct cytotoxic effects. BPA was also found in the urine of 394 tested US adults with a mean result of 1.33µg/L (Calafat et al., 2005.)

More than simply assessing the effect of a low concentration of BPA on mammalian cells in vitro, it was decided to ascertain if there was a particular concentration (or concentrations, in fact) of BPA that showed markedly increased cell cytotoxicity. To investigate a possible dose-dependant relationship, it was decided to use tenfold dilutions from $4x10^{-4}$M BPA down to extremely low $4x10^{-9}$M BPA. By plotting the results of all the values inbetween, it was hoped that a clear jump in toxicity would make itself clear.
Alternatively, a spread of concentrations would also elucidate other facts about BPA even if the expected dose-dependant relationship was found to not be the case. For example, if BPA appeared highly toxic at all our tested

ISSN 1754-2383 [Online]

concentrations, this would also be a valid result, although testing of concentrations outside those chosen would still be in order. Again, if BPA showed only low toxicity at all concentrations, this should be furthered by testing at other concentrations also to make sure that BPA had a standard cytotoxicity over a wide range of concentrations (although this scenario was deemed to be highly unlikely).

The results of the experiment will provide insight into the effect of BPA on cellular viability in mammalian cells when there is no interference from in-vivo metabolic processes, as previously mentioned. It should also be noted that in-vitro studies do away with differences in health, genetics, age and a host of other factors, allowing a clear cut result to be obtained that is both independent of all these factors but, more importantly, is far more repeatable and, ideally, potentially usable as a basic benchmark for BPA exposure and toxicity in experiments or diagnostics to come.

2. Materials and Methods

2.1 Cell culture maintenance and passaging

Chinese hamster ovary (CHO-K1) cells were cultured in Ham's F-12 medium supplemented with 10% foetal bovine serum and 2mM glutamine and seeded at a concentration of $5x10^4$ cells/ml in 8ml of medium in culture flasks. At each passage, three new flasks were seeded at this same concentration. Cells were kept healthy by passage and re-seeding in this manner twice a week (on Tuesdays and Fridays, unless insufficient confluence required a delay.)

Passages involved first selecting which vial of the previous three was not contaminated and showed best confluence to around 90% confluence. This was achieved simply by reverse microscopy and comparison by eye. The method of seeding the other vials from this chosen source involved pouring off medium, washing twice with 5ml PBS (phosphate buffer saline) solution and then trypsinising with 1ml 0.25% trypsin. Trypsin was washed over the surface of the cells simply by tilting the vial for around 20 seconds and then poured off immediately. Cells were then left to detach from the vial and from each other until they had a rounded appearance when viewed under reverse microscopy. Cells were agitated by hand tapping of vial to assist in detaching them from the base of the vial.

New medium was then added (5ml) and the cells resuspended. Cell counts were carried out manually by haemocytometer. From this known concentration of cells per ml, an appropriate volume of cell suspension to provide the correct number of cells to seed a new vial was determined. Medium was added to the new vials first so as to prevent the clumping of cells that would have occurred if cells were added to a dry vial before medium. Three such vials were prepared and labelled after each passage and then stored at 37^{o}C until the next passage in an incubator.

Example calculation (taken from passage performed on 04/12/2007):

Cells found in each quadrant of haemocytometer =
60, 49, 75, 90

Mean cells per quadrant = 68.5
Cells per $0.1mm^3$ = 68.5
Cells per $1mm^3$ = 685
Cells per $1cm^3$ (cells per 1ml)= 685000 = $6.85x10^5$ cells

To make up $4x10^5$ cells total in the vial, this corresponds to ($4x10^5$ / $6.85x10^5$) = 0.583ml of our suspension of cells.

Therefore the new vial should be seeded with:

0.583ml cell suspension + 7.417ml medium = 8ml

BPA solution was prepared by dissolving 0.0023g BPA in 50µl DMSO. This was then made up to 100ml by addition of 99.95ml CHO medium. The original concentration of this made up stock solution of BPA in DMSO and medium was $1x10^{-4}$M BPA. This was then made in tenfold dilutions to $1x10^{-5}$M through $1x10^{-9}$M in the following manner:

Concentration (BPA) desired	BPA added (ml)	Medium added (ml)
$1x10^{-4}$M	5 of stock solution	0
$1x10^{-5}$M	0.5 of $1x10^{-4}$M solution	4.5
$1x10^{-6}$M	0.5 of $1x10^{-5}$M solution	4.5
$1x10^{-7}$M	0.5 of $1x10^{-6}$M solution	4.5
$1x10^{-8}$M	0.5 of $1x10^{-7}$M solution	4.5
$1x10^{-9}$M	0.5 of $1x10^{-8}$M solution	4.5

All of these were stored in foil-wrapped bougies at 3^{o}C.

DMSO and medium control was prepared to a 1:2000 dilution by adding 1µl DMSO to 1999µl medium. Neutral red stock solution was prepared at 700µg ml^{-1} by adding 0.07g of neutral red powder to 100ml of PBS and stored in a sterile brown vial at 3^{o}C. For the neutral red assay, a working solution was taken from this and made to 70µg ml^{-1} by tenfold diluting in CHO medium. Acidified ethanol control was made by adding 50ml ethanol, 1ml acetic acid and 49ml distilled water in a glass bottle and stored in refrigerator at 3^{o}C.

ISSN 1754-2383 [Online]

2.2 Neutral Red Assay

A sterile 96 well plate was set up in the configuration shown in figure 1. Each well was first seeded with cells following a method similar to a regular passage. However, two flasks of cells were emptied of medium, washed twice with PBS, trypsinised and resuspended in 5ml medium and one poured into the other due to greater number of cells needed for 96 well plate. A haemocytometer count was performed as before. This time, a concentration of $2x10^4$ cells / cm^3 was required. As per figure 1, 44 wells were to be filled, each with 200µl of cell suspension at $2x10^4$ cells / cm^3. This required a minimum of (0.2ml x 44 wells) = 8.8ml of cell suspension. 10ml of suspension was made at a concentration of $2x10^4$ cells / cm^3 to make way for error.

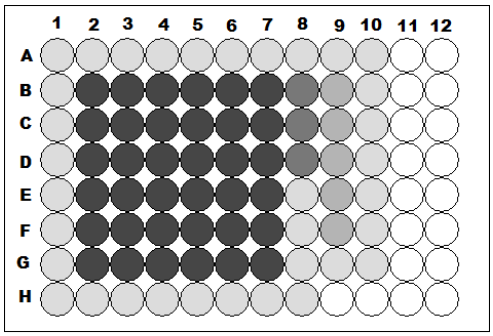

Figure 1: Configuration of 96 well microplate for Neutral Red Uptake Assay

= BPA **=Acidified ethanol**
= DMSO+MEDIUM **= Medium**

After the wells were each seeded with 200µl of this suspension, blank CHO medium was added to all surrounding wells to act as a heat sink. The plate was then incubated for 24 hours at 37°C.

Immediately after this, medium was removed by pipette and replaced with test substances (BPA in DMSO in medium, DMSO in medium and acidified ethanol solution for their respective wells as per figure 1). This was incubated for 48 hours at 37°C.

Following this exposure, all supernatants (except heat sink wells) were removed by pipette. 200µl of the 70µg/ml neutral red in medium solution was then added to all test wells and the plate again incubated at 37°C for three hours to allow uptake of the stain. After this, all non-heat-sink wells were drained of NR-containing medium by pipette and then washed thrice with 200µl PBS each. After the addition of PBS to the wells for each wash, a gentle spin of the plate for 1min was performed to assist in washing the cells. Finally, after the removal of the last washing PBS, to release the NR taken up by the cells, each well had 200µl acidified ethanol added to it to lyse the cells and the plate spun again for 1min to mix well. The plate was then read for absorbance at 540nm using an OPTImax tunable microplate reader (Molecular Devices) and the results recorded using SOFTMAX software (Molecular Devices). It was decided to use one-way ANOVAs to test for significance between treatments of BPA because only one variable was in question here – the agent to which the cells were exposed. This was then used to find a point at which cytotoxicity showed a marked increase. Analyses such as this were also carried out to find differences between treatments and controls.

3. Results

Results of the neutral red assay and associated controls are shown in figure 2. Figure 3 shows stacked data of concentrations $1x10^{-4}$M to $1x10^{-6}$M BPA on the left and $1x10^{-7}$M to $1x10^{-9}$M BPA on the right.

Mean absorbance for $1x10^{-4}$M BPA was 1.924 (SD 0.253, SEM 0.103). Mean absorbance for $1x10^{-5}$M BPA was less, at 1.905 (SD 0.177, SEM 0.072).

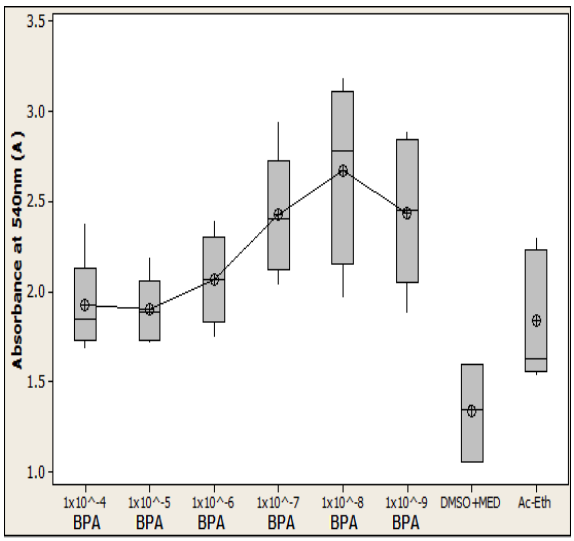

Figure 2: Boxplots of absorbance data from Neutral Red Uptake Assay. DMSO+MED = DMSO and medium control, Ac-Eth = acidified ethanol control.

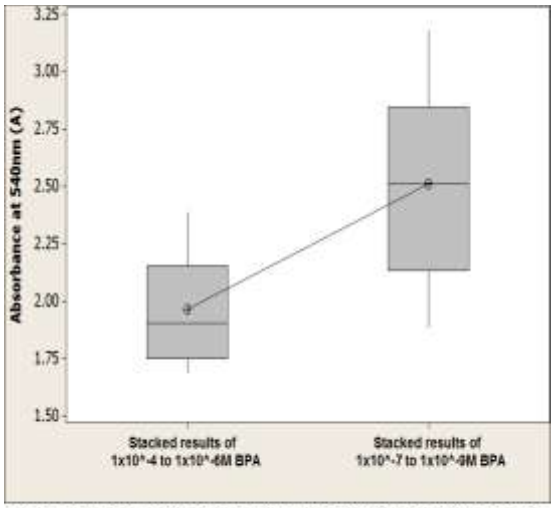

Figure 3: Boxplot of stacked results of 1x10^-4M to 1x10^-6M BPA versus stacked results of 1x10^-7M to 1x10^-9M BPA. One-way ANOVA shows clearly significant difference (p=0.000). However, there was no statistically significant difference by one-way ANOVA within the groups (p=0.421 and p=0.526 for higher 3 concentrations (left boxplot) and lower 3 concentrations (right boxplot) groups respectively.)

ISSN 1754-2383 [Online]

Mean absorbance of $1x10^{-6}$M BPA was 2.068 (SD 0.246, SEM 0.100), considerably less than that for $1x10^{-7}$M BPA, which was 2.430 (SD 0.333, SEM 0.136). This area is where most of the interest lies. There was then only a small increase in mean absorbance of 0.239A to 2.669 for $1x10^{-8}$M BPA (SD 0.481, SEM 0.196). The lowest concentration of BPA tested, $1x10^{-9}$M BPA, had a mean absorbance of 2.435 (SD 0.396, SEM 0.162), which is actually less than the previous mean absorbance by 0.234A. There is, however, no statistically significant difference here between $1x10^{-8}$M BPA treatment and $1x10^{-9}$M BPA treatment (p=0.381).

The DMSO + medium control had a mean absorbance of 1.333 (SD 0.272, SEM 0.157), which is statistically significantly lower than all the means for the BPA treatments (p=0.001 for a one-way ANOVA between stacked results of all BPA treatments versus the DMSO + medium control.) The acidified ethanol control had a mean absorbance considerably higher than that of the DMSO + medium control by a margin of 0.509A, coming out at 1.842 (SD 0.360, SEM 0.161).

There was no statistically significant difference between the concentrations of $1x10^{-4}$M to $1x10^{-6}$M BPA (p=0.421). Neither was there a significant difference between $1x10^{-7}$M to $1x10^{-9}$M BPA (p=0.526).

There was a highly statistically significant difference between the two measured extreme concentrations of $1x10^{-4}$ and $1x10^{-9}$M BPA (p=0.024), satisfying the 95% confidence interval. When results are taken as groups, there was an extremely statistically significant difference between $1x10^{-4}$ to $1x10^{-6}$M BPA and $1x10^{-7}$ to $1x10^{-9}$M BPA (p=0.000), satisfying the 99.9% confidence interval (stacked one-way ANOVA of $1x10^{-4}$ to $1x10^{-6}$M BPA versus $1x10^{-7}$ to $1x10^{-9}$M BPA shown in figure 3.)

A significant difference exists also between $1x10^{-5}$M and $1x10^{-8}$M BPA (p=0.004). Significance is not found, however, between $1x10^{-6}$ M and $1x10^{-7}$M BPA (p=0.058) as this is just short of the 95% confidence interval.

It is worth noting that there is a noticeable difference in the boxplot between the DMSO + medium control and the acidified ethanol control. These are not statistically significant (p=0.081), falling short of the 95% confidence interval (figure 2.) Also it is worth noting that the DMSO + medium control shows greatly decreased absorbance compared to all BPA treatments and even acidified ethanol. Possible reasons for this, both experimentally and toxicologically, will be elaborated upon in the discussion.

4. Discussion

There is a clear progression from lower absorbances (lower cellular viability) to higher absorbances (higher cellular viability) as the concentration of BPA decreases from left to right in figure 2. The first main point of interest however, lies in discerning at what level there is greatest change in cellular viability and therefore at what level cells begin to experience the largest drop in viability for the smallest increase in concentration. This could be seen as the point of tolerance or toxicity “threshold”. The second main point of interest is in discerning to what level even the lower concentrations of BPA affect cellular viability compared to control.

In order to find out at what point there is greatest increase in cytotoxic effect of BPA for the smallest increase in BPA concentration, the lowest and highest concentrations used were compared for statistically significant difference and were found to be statistically different (p=0.024). Then the next highest and next lowest would be compared until there was no statistically significantly difference between the cytotoxic effect of those concentrations, at which point it could be said that around or between those two concentrations lay the point of greatest cytotoxic effect for smallest change in concentration. Comparison of $1x10^{-5}$M and $1x10^{-8}$M BPA revealed highly significant difference also (p=0.004). It was only when our middle two concentrations of $1x10^{-6}$ and $1x10^{-7}$ were compared that we were unable to satisfy a 95% confidence interval (p=0.058).

The significance level drops below the 95% confidence interval only when making comparison between the $1x10^{-6}$ M and $1x10^{-7}$M BPA measurements; it is therefore in that range where differences become more difficult to discern. It is also visible from the boxplots in figure 2 that the greatest change in toxicity exists between the $1x10^{-6}$ M and $1x10^{-7}$M BPA tests as visible by the large gradient of the line between the two. It is therefore reasonable to suggest that this is indeed the point at which the smallest increase in concentration produced the biggest increase in cytotoxicity. Further experiments with smaller differences in concentration, but all within the $1x10^{-6}$M and $1x10^{-7}$M BPA range could continue to pinpoint the concentration at which these mammalian cells start to exhibit a pronounced reduction in cell viability. It is clear to see, however, that, in conclusion, BPA does indeed have cytotoxic effect, observable in vitro, and that even low concentrations of around $1x10^{-6}$M to $1x10^{-7}$M BPA have a pronounced effect on cell viability.

It is unfortunate, however, that the absorbances for the control DMSO + medium were so unexpectedly low (for reasons hypothesised below) as this would have allowed for all results to be compared to a “healthy” control. Whilst it was known that DMSO has a weakly cytotoxic effect, it was completely unanticipated that the control would give such an outlying result.

Regarding such apparent high cytotoxicity of DMSO + medium controls, the exact cause of this unexpected reading is unknown. It was expected that DMSO + medium would produce higher absorbances (better cell viability) than all BPA exposures and acidified ethanol 100% lysis control.

Whilst the experiment's meaning remains intact regarding the cytotoxic effects of relative concentrations of BPA, it is suggested that the experiment be repeated with many more repeats of the DMSO + medium control. This would give a much more definite answer than just the three that were available here. There was also some concern over

ISSN 1754-2383 [Online]

the expansion of DMSO when exposed to the incubator's temperature. It is particularly strange that the acidified ethanol control seemed to produce far less cellular lysis than expected. Whilst the acidified ethanol stock solution was left in the refrigerator for some time, it was not considered to be one of the agents that would deteriorate.

Therefore it would be worth repeating the DMSO + medium controls with a far greater number of repeats and away from all other wells in case of expansion overflow and also to perform fresh acidified ethanol lysis of the same concentration of seeded CHO-K1 cells in wells to ascertain whether or not this reading is an anomaly, due to settling or deterioration of stock solution or some other mechanism.

Finally, one last area of improvement for studies such as this would be to use non polycarbonate wares for all of the experiment to eliminate BPA leaching. As a great deal of equipment here (most notably, the vials and microplates) were made of polycarbonate plastic, there will most likely have been some leaching of BPA from the equipment. Whist this can be ignored to some extent due to the fact that the same equipment was used under the same conditions for all of the work, there may well have been some effect as this work's very nature elucidates that BPA has cytotoxic effects in-vitro at even very low concentrations. The presence of a solving agent such as DMSO may well have added to the leaching as solvents are known to increase the rate of BPA leaching (Krishnan et al., 1993.)

In conclusion, there are far wider implications of this study than simply that BPA is cytotoxic at low concentrations. A re-evaluation of the safety of polycarbonate plastic is in order. At such low concentrations of BPA as $1x10^{-6}$M (micromolar), it is clear to see, coupled with the fact that polycarbonate plastics and BPA leachates are all around us, that this can have a definite and direct cytotoxic effect. It is worth noting, however, that published literature cited here has not found amounts of BPA present in human fluids that cross the putative "threshold of toxicity" found here. Further studies, particularly with the addition of more controls, are in order to more accurately elucidate the exact effects at different concentrations.

What most definitely is cause for concern, however, is the amount of BPA leached from aging polycarbonate plastic. The study by Howdeshell et al (2003) clearly shows a value of 310µg/L leaching from aged polycarbonate animal cages into distilled water. This concentration is above the "threshold of toxicity" as it corresponds to $1.36x10^{-6}$M BPA. Therefore, rather than concentrating on reassuring statistics that show low physiological levels of BPA, studies must begin into which polycarbonate plastic products (including dental resins as BPA leaches from these also (Takahashi & Oishi, 2000)) and their BPA leaching curves. For example, there may well become a point in the lifetime of certain products at which the levels of BPA leaching cross this threshold of toxicity.

In addition to the known oestrogenicity of BPA, the cytotoxic effects of directly ingesting such levels of BPA are as yet unknown. More studies need conducting into how BPA is absorbed as, while serum levels and urine levels may seem low at the moment, there may be much higher levels directly acting on the gastrointestinal tract as this is what is in contact with ingested fluids, for example. This could, in turn lead to increased risk of GIT pathology. This may also be true of the liver which is responsible for the conjugation of BPA and may well accumulate there at higher levels than found in serum.

This becomes even more concerning when bearing in mind that levels of BPA shown to be cytotoxic will be released in relatively large amounts from polycarbonate baby bottles and these are warmed for the baby, further increasing BPA leaching. Due to the reduced capacity of babies (by pure lack of body mass) to eliminate BPA, the cytotoxic effects of this compound may well be aggravated due to the well-appreciated dose/body mass relationship. These thoughts make the cytotoxicity of these very low concentrations of BPA all the more worrying and strengthen existing calls for a thorough investigation into the distribution of BPA presence throughout the human body. This must be joined by studies into the leaching of BPA from products that will cause ingestion/exposure to BPA and the product age/BPA-leaching relationship and a more extensive investigation into the occurrence of BPA in our environment and therefore its effects on the wider ecosystem.

Acknowledgements

The author would like to thank Dr. Awadhesh Jha (supervision), Awantha Dissanayake (lab technical support), Lynne Cooper (cell culture methodology) and Sarah Jamieson (lab technical support) at the University of Plymouth.

References

Alonso-Magdalena P, Morimoto S et al., 2006, "The Estrogenic Effect of Bisphenol A Disrupts Pancreatic β-Cell Function In-Vivo and Induces Insulin Resistance", Environ Health Perspect, 114, pp106-112.

Bergeron RM, Thompson TB et al., 1999, "Estrogenicity of bisphenol A in a human endometrial carcinoma cell line", Mol Cell Endocrinol, 25;150, pp179-187.

Calafat AM, & Kuklenyik Z et al., 2005, "Urinary Concentrations of Bisphenol A and 4-Nonylphenol in a Human Reference Population", Environ Health Perspect, Vol 113, No 4, pp391-395.

Fox JE, Gulledge J et al., 2007, "Pesticides reduce symbiotic efficiency of nitrogen-fixing rhizobia and host plants" Proc Nat Acad Sci USA, 104;24, pp10282-10287.

Honma S, Suzuki A et al., 2002, "Low dose effect of in utero exposure to bisphenol A and diesthylstilbestrol on female mouse reproduction", Reprod Toxicol, 16;2, pp117-122.

Howdeshell K, Hotchkiss AK et al., 1999, "Environmental

toxins: Exposure to Bisphenol A Advances Puberty", Nature, 401, pp763-764.

Howdeshell KL, Peterman PH et al., 2003, “Bisphenol A is Released from Used Polycarbonate Cages into Water at Room Temperature”, Environ Health Perspect, 111, pp1180-1187.

Hunt PA, Koehler KE et al., 2003, “Bisphenol A Exposure Causes Meiotic Aneuploidy in the Female Mouse”, Curr Biol, 13, pp546-553.

Ikezuki Y, Tsutumi O et al., 2002, “Determination of bisphenol A concentrations in human biological fluids reveals significant early prenatal exposure”, Hum Reprod, 17;11, pp2839-2841.

Ishido M, Masuo Y et al., 2004, "Bisphenol A causes hyperactivity in the rat concomitantly with impairment of tyrosine hydroxylase immunoreactivity", J Neurosci Res, 76, pp423-433.

Kawagoshi Y, Fujita Y et al., 2003, “Estrogenic chemicals and estrogenic activity in leachate from municipal waste landfill determined by yeast two-hybrid assay”, J Environ Monit, 5, pp269-274.

Kawai K, Nozaki T et al., 2003, “Aggressive Behavior and Serum Testosterone Concentration during the Maturation Process of Male Mice: The Effects of Fetal Exposure to Bisphenol A”, Environ Health Perspect, 111, pp175-178.

Krishnan A, Stathis P et al., 1993, “Bisphenol-A: An estrogenic substance is released from polycarbonate flasks during autoclaving”, Endocrinology, 132;6, pp2279-2286.

Masuno H, Kidani T et al., 2002, “Bisphenol A in combination with insulin can accelerate the conversion of 3T3-L1 fibroblasts to adipocytes”, J Lipid Res, 43, pp676-684.

Moriyama K, Tagami T et al., 2002, “Thyroid Hormone Action Is Disrupted by Bisphenol A as an Antagonist”, The J Clin Endocrinol Metab, 87, pp5185-5190.

Murray TJ, Maffini MV et al., 2007, “Induction of mammary gland ductal hyperplasias and carcinoma in situ following fetal bisphenol A exposure”, Reprod Toxicol, 23, pp383-390.

Nagel SC, vom Saal FS et al., 1997, “Relative Binding Affinity-Serum Modified Access (RBA-SMA) Assay Predicts the Relative In-vivo Bioactivity of the Xenoestrogens Bisphenol A and Octylphenol”, Environ Health Perspect, 105, pp70-76.

Negishi T, Kawasaki K et al., 2004, "Behavioural Alterations in Response to Fear-Provoking Stimuli and Tranylcypromine Induced by Perinatal Exposure to Bisphenol A and Nonylphenol in Rats", Environ Health Perspect, 112, pp1159-1164.

Ouchi K & Watanabe S, 2002, "Measurements of bisphenol A in human urine using liquid chromatography with multi-channel coulometric electrochemical detection", J Chromatogr, 780, pp365-370.

vom Saal FS & Hughes C, 2005, “An extensive new literature concerning low-dose effects of bisphenol A shows the need for a new risk assessment”, Environ Health Perspect, 113;8, pp1883-1887.

Sawai C, Anderson K et al., 2003, "Effect of bisphenol A on Murine Immune Function: Modulation of Interferon-gamma, IgG2a, and Disease Symptoms in NZB x NZW F1 Mice", Environ Health Perspect, 111, pp1883-1887.

Schönfelder G, Wittfoht W et al., 2002, “Parent bisphenol A accumulation in the human-maternal-fetal-placental unit”, Environ Health Perspect, 110;11, pp703-707.

Takahashi O, & Oishi S, 2000, “Disposition of Orally Administered 2,2-Bis(4-hydroxyphenyl)propane (Bisphenol A) in Pregnant Rats and the Placental Transfer to Fetuses”, Environ Health Perspect, 108:10, pp931-935.

Vandenberg LN, Maffini MV et al., 2007, “Exposure to Environmentally Relevant Doses of the Xenoestrogen Bisphenol-A Alters Development of the Fetal Mouse Mammary Gland”, Endocrinology, 148, pp116-127.

Volkel W, Colnot T et al., 2002, “Metabolism and kinetics of bisphenol A in humans at low doses following oral administration”, Chem Res Toxicol, 15, pp1281-1287.

Yoshino S, Yamaki K et al., 2004, "Prenatal exposure to bisphenol A up-regulates immune responses, including T helper 1 and T helper 2 responses, in mice", Immunology, 112, pp489-495.

Zoeller RT, Bansal R et al., 2005, "Bisphenol-A, an Environmental Contaminant that Acts as a Thyroid Hormone Antagonist in Vitro, Increases Serum Thyroxine, and Alters RC3/Neurogranin Expression in the Developing Rat Brain", Endocrinology, 146, pp607-612.

ISSN 1754-2383 [Online]